J. Clin. Chem. Clin. Biochem.
Vol. 21, 1983, pp. 417–427

A Comparative Study of Five Different Methods for the Determination of 3-Methoxy-4-hydroxymandelic Acid in Urine

By *C. G. Thomasson*[1]), *B. G. Blijenberg*

Department of Clinical Chemistry, University Hospital Rotterdam Dijkzigt, and *Department of Chemical Pathology, Erasmus University, Rotterdam, The Netherlands*

G. A. M. Eilers

Institute of Biostatistics, Erasmus University, Rotterdam, The Netherlands and

B. Leijnse

Department of Clinical Chemistry, University Hospital Rotterdam Dijkzigt, and *Department of Chemical Pathology, Erasmus University, Rotterdam, The Netherlands*

(Received September 22/December 27, 1982)

Summary: Five different methods for the determination of 3-methoxy-4-hydroxymandelic acid (vanilmandelic acid, VMA) in urine were compared: a GLC-FID catecholamine metabolite profiling method, an HPLC method with electrochemical detection, the method of *Pisano* et al. ((1962) Clin. Chim. Acta *7*, 285–291), a one dimensional paper chromatographic method with diazotized *p*-nitroaniline staining and the commercially available Bio-Rad VMA by Column Test. The comparison consisted of an imprecision study, a linearity check, a recovery study, a split sample comparison and an interference study. The best results of the imprecision study (n = 8) were found with the Bio-Rad and the HPLC method (within-run imprecision had a coefficient of variation (CV) of 5.1% and 1.4%; between-days CV of 5.9% and 6.0% respectively for values of 32.4 µmol/l and 24.5 µmol/l). The *Pisano* method had the poorest within-run CV (14.6%) and between-days CV (16.8%) for a value of 23.2 µmol/l. All methods showed good linearity. The mean recovery of the HPLC method was 101.3%; the mean recovery of the other four methods ranged from 93.9%–96.0%. The split sample comparison showed that the accuracy of the HPLC, the GLC and the *Pisano* method is comparable. The accuracy of the paper chromatographic method and the Bio-Rad method had a positive bias compared with the HPLC method. Especially the positive bias of the Bio-Rad method can be very large. The HPLC method was not influenced by the compounds tested in the interference study, whereas the GLC method in some cases only suffered from overloading problems. The *Pisano* and the Bio-Rad method were most influenced by the interfering compounds tested. We conclude that the HPLC and the GLC methods are superior to the other three VMA methods. From an analytical point of view HPLC is the method of choice for determining 3-methoxy-4-hydroxymandelic acid in urine.

Vergleichende Untersuchung von fünf verschiedenen Methoden zur Bestimmung von 3-Methoxy-4-hydroxymandelsäure im Harn

Zusammenfassung:

Fünf verschiedene Methoden zur Bestimmung von 3-Methoxy-4-hydroxymandelsäure (Vanillinmandelsäure, VMA) im Harn werden verglichen:

1. Gaschromatographische Methode mit Flammenionisations-Detektion zur Erstellung von Catecholaminmetabolit-Profilen.

[1]) Present adress: Algemeen Ziekenhuis "de Tjongersschans", Thialfweg 44, NL-8441 PW Heerenveen, The Netherlands.

2. Hochleistungsflüssigchromatographische (HPLC) Methode mit elektrochemischer Detektion.
3. Methode nach *Pisano* ((1962) Clin. Chim. Acta 7, 285–291).
4. Eindimensionale papierchromatographische Methode mit Detektion durch diazotiertes *p*-Nitroanilin.
5. Säulen-Test Bio-Rad VMA.

Der Vergleich umfaßte:

a) Untersuchungen zur Präzision (n = 8)

Die besten Ergebnisse wurden mit der Bio-Rad und der HPLC-Methode erzielt: VK in der Serie 5,1% und 1,4%; VK von Tag zu Tag 5,9% und 6,0%; c = 32,4 und 24,5 µmol/l. Die *Pisano*-Methode hatte die schlechteste Präzision: VK in der Serie 14,6%; VK von Tag zu Tag 16,8%; c = 23,2 µmol/l.

b) Linearitätsprüfung

Alle Methoden wiesen eine gute Linearität auf.

c) Untersuchungen zur Wiederfindung

Die mittlere Wiederfindung der HPLC-Methode betrug 101,3%, die der anderen 4 Methoden 93,9–96,0%.

d) Prüfung der Richtigkeit durch Vergleich an der geteilten Probe

Die Richtigkeit von HPLC-, gaschromatographischer und *Pisano*-Methode ist vergleichbar. Papierchromatographische und Bio-Rad-Methode zeigten, verglichen mit der HPLC-Methode, eine positive Abweichung, die besonders bei der Bio-Rad-Methode sehr hoch sein kann.

e) Untersuchungen von Störeinflüssen

Die HPLC-Methode wurde durch die geprüften Verbindungen nicht gestört, während die gaschromatographische Methode manchmal unter Überladungsproblemen litt. Die *Pisano*- und die Bio-Rad-Methode zeigten die häufigsten Störungen durch die geprüften Substanzen.

Schlußfolgerung: HPLC und Gaschromatographie sind zur Bestimmung von 3-Methoxy-4-hydroxymandelsäure vorteilhafter als die anderen drei Methoden. Vom analytischen Standpunkt ist die HPLC die Methode der Wahl zur Bestimmung von 3-Methoxy-4-hydroxymandelsäure im Harn.

Introduction

In 1957 *Armstrong* et. al. (1, 2) discovered that the excretion of 3-methoxy-4-hydroxymandelic acid (vanilmandelic acid) is increased in the urine of pheochromocytoma patients. Nowadays we know that it is one of the main metabolites of the catecholamines norepinephrine and epinephrine. Together with 3-methoxy-4-hydroxyphenylethyleneglycol it accounts for about 80% of the overall turnover of norepinephrine and epinephrine (3). Its determination, together with other urinary catecholamine metabolites is important in the diagnosis and the follow up of patients with neural crest tumors (neuroblastoma, ganglioneuroblastoma and pheochromocytoma).

Amstrong et al. determined the urinary excretion of 3-methoxy-4-hydroxymandelic acid with a method based on organic solvent extraction, two dimensional paper chromatography and staining with diazotised *p*-nitroaniline (4). In the years following their discovery other quantitative methods were described (5–7). One of these, the method of *Pisano* et al. (7), based on organic solvent extraction and photometry of vanillin, the oxidation product of vanilmandelic acid, is still in use in many clinical chemical laboratories. In the last decade several articles have been published concerning the results of 3-methoxy-4-hydroxymandelic acid determinations using gas liquid chromatography (GLC) with flame ionisation detection (8–15) or electron capture detection (16, 17). More recently high performance liquid chromatography (HPLC) procedures with ultra violet detection (18, 19), fluorescence detection (20, 21), electrochemical detection (22–27) or a periodate oxidative monitor (28, 29) were described. As stated by the authors, the reasons for using GLC or HPLC are: saving of time, possibility of multicomponent determination and improvement of precision, sensitivity and specificity. Although theoretically this last argument may be valid, little practical evidence is presented. In only half of the articles cited, the authors compared their GLC or HPLC method with a classical one, mostly the *Pisano* method (8, 10, 15, 20, 22,

23, 27–29) and sometimes a two dimensional paper chromatographic method (18, 19). In merely half of the cited articles imprecision data are given (10–12, 14, 15, 20, 23, 25, 27–29). With the exception of l.c. (25), these articles fail to present imprecision data of the classical vanilmandelic acid method when a split sample comparison was carried out. This lack of quality control and split sample comparison data make it difficult to compare different 3-methoxy-4-hydroxymandelic acid methods. Therefore we made a comparison, including these items, between two modern 3-methoxy-4-hydroxymandelic acid procedures (the GLC catecholamine metabolite profiling method described by *Muskiet* et al. (14) and a modification by *Moleman & Borstrok* (30) of the HPLC method of *Morrisey & Shihabi* (23)), two classical 3-methoxy-4-hydroxymandelic acid determinations (the method of *Pisano* et. al. (7) and an one dimensional paper chromatographic procedure comparable with the method described by *Vahidi* (31)) and one commercially available 3-methoxy-4-hydroxymandelic acid test (the Bio-Rad VMA by Column Test).

It is known that for a urinary 3-methoxy-4-hydroxymandelic acid determination the patient must keep to a certain diet and stop medication. Therefore we were also interested in establishing the influence of various compounds which are known to interfere in the different methods in a methodological way, according to the compilation of *Young* et al. (32). This investigation together with the comparison study was carried out by mailing urine samples to five different laboratories, each performing one of the five methods mentioned above and having sufficient experience with this method.

Materials and Methods

The following chemicals, 3-methoxy-4-hydroxymandelic acid standard, control urine and urine samples were used for preparing different samples of the comparison study.

Chemicals

4-Hydroxy-3-methoxymandelic acid (vanilmandelic acid, VMA), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA), *p*-hydroxymandelic acid, 4-hydroxy-3-methoxybenzoic acid (vanillic acid) and salicyluric acid were purchased from Sigma Chemical Co., St. Louis, M.O. 63178.

2,5-Dihydroxybenzoic acid (gentisic acid), *p*-aminosalicylic acid, 5-hydroxy-indoleacetic acid, glycerylguaiacolate and tetrabromophenolsulphonephthalein (Bromthalein) were from Merck, Darmstadt, FRG.

2,5-Dihydroxyphenylacetic acid (homogentisic acid) and phenolsulphonephthalein were from Fluka AG, CH-9470 Buchs, Switzerland.

3,4-Dihydroxyphenylacetic acid was from Labkemi, Stockholm, Sweden.

3-Methoxy-4-hydroxymandelic acid standard

A 100 µmol/l standard was prepared in 0.05 mol/l hydrochloric acid solution and in part diluted to 50 µmol/l with 0.05 mol/l hydrochloric acid solution.

Control urine

The lyophilized control urine I (lot. No. 090V02) and control urine II (lot. No. 1V214) both from Ortho Diagnostic Inc. Rariton, New Jersey 08869, were reconstituted with distilled water. A mixture of 30 vials of control urine I and 9 vials of control urine II was aliquoted and stored at −20 °C. This mixture was used as a control urine.

Urine samples

All urine samples, collected after the patients had kept to a diet (no coffee, tea, cola drinks, bananas, vanillin and products flavoured with vanillin) were preserved by acidification to pH 1.0 with hydrochloric acid. The samples were aliquoted and stored at −20 °C until they were distributed.

Sample distribution

During a six month period we distributed by mail every two weeks a series of urine samples. In total 12 series containing 106 samples were distributed. The urine samples in each series were randomized using a table of permutations. On arrival the thawed samples were stored at 4 °C when the 3-methoxy-4-hydroxymandelic acid determination could be performed within two days. Otherwise the samples were frozen.

Imprecision study and split sample comparison

Twelve different urine samples were divided in two groups of six samples. Each group, completed with two samples of the control urine, was distributed four times, resulting in eight series. The results of these sixtyfour 3-methoxy-4-hydroxymandelic acid determinations, four determinations in twelve different urine samples and eight determinations in duplicate of the control urine, were used for the split sample comparison and the imprecision study.

Linearity check

Linearity was checked with a series of nine samples, consisting of a urine with a low (± 10 µmol/l) and one urine with a high (± 100 µmol/l) 3-methoxy-4-hydroxymandelic acid content and mixtures of these two urines having high/low ratios (by volume) of 1/7, 2/6, 3/5, 4/4, 5/3, 6/2 and 7/1.

Recovery study

For the recovery study the same urine sample with a low content from the linearity check was enriched with the following 3-methoxy-4-hydroxymandelic acid concentrations: 20, 40, 60, 80 and 100 µmol/l urine.

Interference study

A pool urine was aliquoted and to separate aliquots one of the following compounds was added per liter of pool urine: 2000 mg of homogentisic acid; 1000 mg of gentisic acid, homovanillic acid, vanillic acid, salicyluric acid, glycerylguaiacolate, *p*-aminosalicylic acid; 500 mg of 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid; 100 mg of *p*-hydroxymandelic acid; 6 mg of phenolsulphonephthalein;350 mg of Bromthalein.

The GLC method

Single determinations were performed with the method according to *Muskiet* et al. (14), with the following modifications:

a) after injection the gas chromatograph was programmed from 120 °C to 200 °C at 16 °C/min followed by 3 °C/min to 260 °C and 15 min at 260 °C.

b) 0.25 ml of n-alkane C_{28} solution (0.5 mmol/l hexane) was added to the urine sample before the extraction with ethyl acetate, as a check for the efficiency of the derivatization of the internal standard propylgallate.

The HPLC method

The HPLC method was a modification by *Moleman & Borstrok*(30) of the procedure of *Morrisey & Shihabi* (23). The following alterations were applied:

a) 1 ml of urine was diluted with 1 ml of 1 mol/l citric acid, containing 5 g EDTA and 5 g $Na_2S_2O_5$ per litre and extracted with 8 ml of ethyl acetate; the ethyl acetate layer was extracted with 4 ml of 0.1 mol/l Na_2HPO_4 pH 8.5, containing 5 g EDTA and 5 g $Na_2S_2O_5$ per litre; after removing the ethyl acetate 50 µl of 700 g/kg $HClO_4$ was added;

b) 50 µl of the acidified phosphate extract was injected;

c) for elution a linear gradient from 2.5 ml to 47.5 ml propanol in 1 litre 0.05 mol/l Na_2HPO_4 (pH 2.7 with $HClO_4$) in 10 min was used with a flow rate of 2 ml/min; the reequilibration time was 5 min;

d) the oxidation potential was maintained at +0.8 V vs. Ag/AgCl;

e) quantification was performed by constructing a calibration curve using four 3-methoxy-4-hydroxymandelic acid concentrations in the range of 10–100 µmol/l, after the extraction described under modification a), based on calculation by area.

The following equipment was used: automatic liquid chromatograph with an autosampler and integrator (Hewlett-Packard 1084 B or Spectra Physics 8100–4100) with an electrochemical detector (Bioanalytical Systems), consisting of an LC–4 controller and a TL–5 cell (glassy carbon electrode and Ag/AgCl reference electrode). The sensitivity was set at 50 nA/V. A filtering of 2 s was used. An analytical column, 15 cm × 4.6 mm I.D., packed with Hypersil ODS, 5 µm particle size, was obtained from Chrompack, Middelburg, The Netherlands. The eluents were filtered through Millipore filters of pore size of 0.45 µm and continuously degassed with helium. The detector response (peak area) and the retention time were calibrated every six samples with a 3-methoxy-4-hydroxymandelic acid standard solution injected directly into the chromatograph. All determinations were performed in duplicate.

In this way it is possible to analyse 30 to 40 urine samples in duplicate in 24 hours.

The lifetime of the reference electrode and the analytical column are about two years. The number of plates are halved during these two years by several thousand determinations.

The *Pisano* method

The method of *Pisano* et al. (7) was performed with one slight modification: all volumes (starting with 2.5 ml of urine) were reduced by half, with the exception of the volumes of the reagents used for the oxidation with $NaIO_4$, the reduction with $Na_2S_2O_5$ and the neutralization with acetic acid and phosphate buffer. The method was performed in duplicate with regard to the unknown; the urine blanks were single determinations.

The Bio-Rad VMA by Column Test

The Bio-Rad VMA by Column Test was performed according to the manufacturer's instructions (33). Only with the determination of the 3-methoxy-4-hydroxymandelic acid standard the concentration of $Na_2S_2O_5$ was 20 times higher than normal. All urine samples were analysed in duplicate. At the end of this comparison study the Bio-Rad VMA by Column Test was no longer available, because of production problems.

The one dimensional paper chromatographic method

The one dimensional paper chromatographic method was developed by *Molenaar* (Department of Chemical Pathology, University of Leiden, The Netherlands) (34) and is comparable with the method published by *Vahidi* et al. (31).

Statistics

Performing two determinations with the control urine, both in duplicate, in the same series, on eight different days, we have the following sources of variance:

$\hat{o}^2_{bd}$: the variance between the eight days (with seven degrees of freedom)

$\hat{o}^2_{wd}$: the variance within each day, between the two determinations (with eight degrees of freedom)

$\hat{o}^2_{du}$: the variance within each day, between the sixteen duplicates (with sixteen degrees of freedom)

We obtained these variances with a two way or three way variance analysis (35) and calculated the within-run and between-days coefficient of variation as follows:

$$\text{within-run CV} = \frac{\sqrt{\hat{o}^2_{wd} + \frac{1}{2}\hat{o}^2_{du}}}{\text{mean}} \times 100\,(\%)$$

(if n = 8 :
8 times 1 determination in duplicate on the same day)

$$\text{between-days CV} = \frac{\sqrt{\hat{o}^2_{bd} + \hat{o}^2_{wd} + \frac{1}{2}\hat{o}^2_{du}}}{\text{mean}} \times 100\,(\%)$$

(if n = 8 :
1 determination in duplicate on 8 days).

Results

Standardization

Table 1 shows the values found for the two distributed 3-methoxy-4-hydroxymandelic acid standards, when each method was performed using its own standard(s) for quantification.

Imprecision study

The results of the between-days imprecision and the within-run imprecision of the five methods are given in table 2. Also the estimates of the variances of three different sources (between-days $\hat{o}^2_{bd}$, within-days between-determinations $\hat{o}^2_{wd}$ and within-days between-duplicates $\hat{o}^2_{du}$) are shown. $\hat{o}^2_{du}$ of the GLC method is not known, because this method was not performed in duplicate.

Linearity check

The results of the linearity check are presented in table 3 as linear regression equations, together with the number of samples (n) and the correlation coefficient (r).

Tab. 1. Values found for two 3-methoxy-4-hydroxymandelic acid standards by the five methods.

	3-methoxy-4-hydroxymandelic acid (μmol/l) found				
μmol/l Standard	GLC	Bio-Rad	*Pisano*	Paper-chromatography	HPLC
50	46.8*)	53.4	50.4	46.0	48.5
100	87.8*)	99.2	103.6	96.0	100.3

*) The standards are taken through the entire analytical procedure and calibration is done with a standard not taken through the entire procedure.

Recovery

In table 4 the results of the recovery experiments are shown. The mean analytical recovery ± 1 SD, together with the results of the linear regression analysis, are given.

Split sample comparison

Figures 1 a—d give the results of the comparison of the 3-methoxy-4-hydroxymandelic acid concentrations found in thirteen urines with all five methods.

Tab. 2. Results of imprecision study with the five methods (n = 8)

	GLC	Bio-Rad	*Pisano**)	Paperchromatography	HPLC
3-methoxy-4-hydroxymandelic acid mean (μmol/)	20.0	32.4	23.2 (24.6)	30.0	24.5
$\hat{\sigma}^2_{du}$	–	0.63	0.60 (0.60)	2.36	0.24
$\hat{\sigma}^2_{wd}$	1.12	2.41	11.22 (1.65)	5.63	0
$\hat{\sigma}^2_{bd}$	6.50	0.94	3.67 (0.31)	5.81	2.01
$\hat{\sigma}^2_{total}$	7.62	3.66	15.19 (2.26)	12.62	2.13
Within-run CV (%)	5.3	5.1	14.6 (5.7)	8.7	1.4
Between-days CV (%)	13.8	5.9	16.8 (6.1)	11.8	6.0

*) Results between brackets are corrected for too high urine blanks.

Tab. 3. Results of the linearity check with the five methods.

For the linear regression analysis: x = 1 is the low, x = 9 is the high urine sample (see Materials and Methods under linearity check); y is the amount of 3-methoxy-4-hydroxymandelic acid found by the five methods; n is the number of urine samples; r is the correlation coefficient.

Method	Linear regression line	n	r
GLC	$y = -1.1 + 8.12\,x$	8	0.9944
Bio-Rad	$y = 11.0 + 9.95\,x$	9	0.9943
Pisano	$y = 1.2 + 10.71\,x$	9	0.9993
Paperchromatography	$y = -2.0 + 12.54\,x$	9	0.9959
HPLC	$y = -3.7 + 11.78\,x$	9	0.9987

Twelve urines were analysed four times and one urine (the control urine of the imprecision study) was analysed sixteen times (eight times in duplicate). The means of these values were used for the linear regression analysis. The results are given with the HPLC method selected as the reference method, because of the good results of the imprecision, linearity, recovery and interference study (see Discussion).

In table 5 we summarized different linear regression lines (no. 1—12), obtained by varying the reference method (HPLC or *Pisano* method) and the concentration unit of 3-methoxy-4-hydroxymandelic acid

Tab. 4. Results of the recovery study with the five methods.

A urine with low 3-methoxy-4-hydroxymandelic acid content was enriched with the following 3-methoxy-4-hydroxymandelic acid concentrations: 20, 40, 60, 80 and 100 μmol/l urine.

For the linear regression analysis: x is the added amount of 3-methoxy-4-hydroxymandelic acid; y is the amount of 3-methoxy-4-hydroxymandelic acid found by the five methods; n is the number of urine samples; r is the correlation coefficient.

Method	Mean recovery ± 1 SD	Linear regression line	n	r
GLC	95.7 ± 9.1	$y = 6.8 + 0.98\,x$	6	0.9932
Bio-Rad	96.0 ± 3.3	$y = 15.0 + 1.00\,x$	5	0.9993
Pisano	94.4 ± 2.1	$y = 6.6 + 0.98\,x$	6	0.9981
Paperchromatography	93.9 ± 2.6	$y = 10.2 + 0.94\,x$	6	0.9990
HPLC	101.3 ± 1.3	$y = 7.5 + 1.01\,x$	6	0.9994

(μmol/l or μmol/24 h) and by correcting for the recovery differences. We also give four linear regression lines found in the literature (no. 13–16).

Interference study

The in vitro influence of twelve compounds on the five methods are presented in table 6. The results are the mean of two determinations.

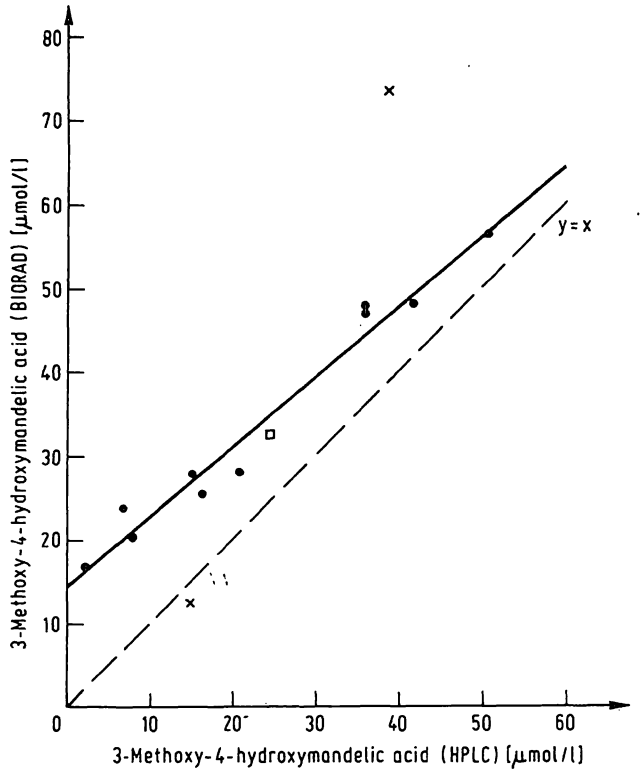

Fig. 1a. Linear regression analysis:
y(Bio-Rad) = 14.5+0.83x (HPLC); n = 11; r = 0.9829.

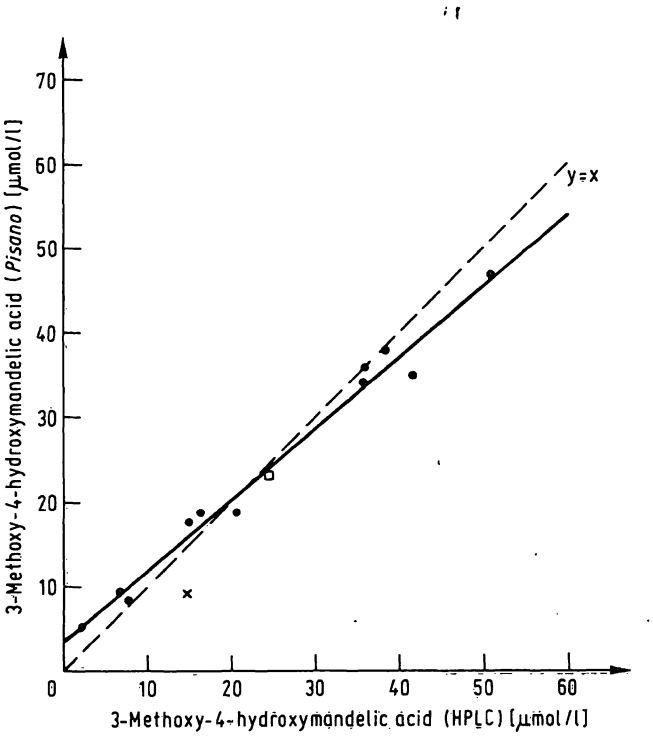

Fig. 1b. Linear regression analysis:
y(*Pisano*) = 3.5 + 0.84 x (HPLC); n = 12; r = 0.9912.

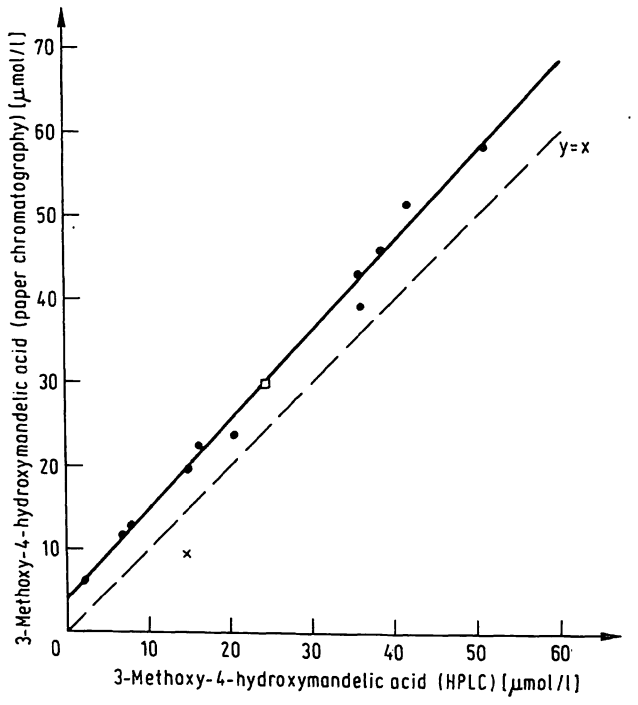

Fig. 1c. Linear regression analysis:
y(Paperchromatography) = 3.9+1.07x (HPLC); n = 12; r = 0.9912.

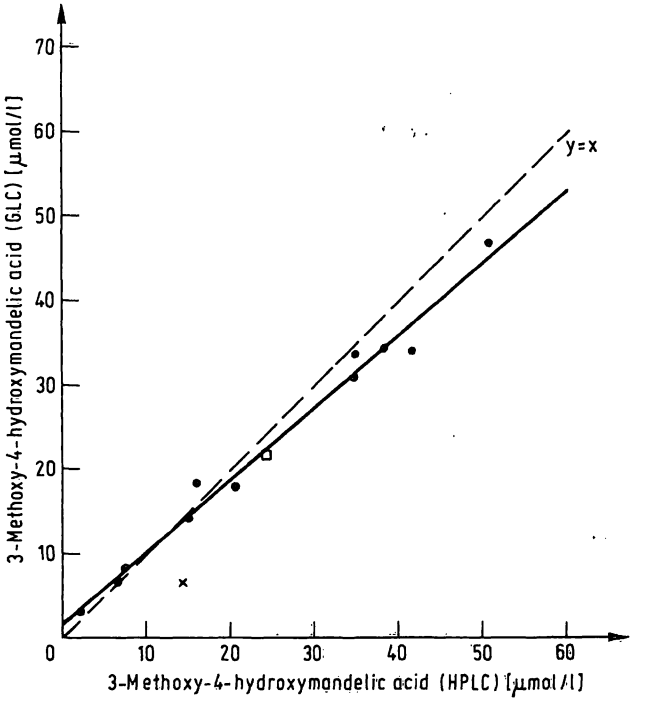

Fig. 1d. Linear regression analysis:
y(GLC) = 1.6 + 0.85x (HPLC); n = 12; r = 0.9929.

Fig. 1a–d. Split sample comparison between the five methods with the HPLC method as reference method (x-axis). Dashed line represents the line y = x; Solid line represents the linear regression line.
● mean of 4 determinations
□ mean of 16 determinations (control urine)
× sample omitted for the linear regression analysis.

Tab. 5. Linear regression lines obtained with the comparison study and by varying the reference method and the concentration unit and by correcting for recovery differences, together with four results from the literature.

No.	Linear regression line	n	r	Concentration unit	Reference
1	y (Bio-Rad) = 14.5 + 0.83 x (HPLC)	11	0.9829	µmol/l	this article
2	y (*Pisano*) = 3.5 + 0.84 x (HPLC)	12	0.9912	µmol/l	this article
3*)	y (*Pisano*) = 3.7 + 0.91 x (HPLC)	12	0.9912	µmol/l	this article
4	y (Pap.chr.) = 3.9 + 1.07 x (HPLC)	12	0.9957	µmol/l	this article
5	y (GLC) = 1.6 + 0.85 x (HPLC)	12	0.9927	µmol/l	this article
6*)	y (GLC) = 1.7 + 0.90 x (HPLC)	12	0.9927	µmol/l	this article
7	y (GLC) = −1.9 + 1.01 x (*Pisano*)	13	0.9959	µmol/l	this article
8	y (GLC) = −2.2 + 0.96 x (*Pisano*)	11	0.9650	µmol/24 h	this article
9	y (HPLC) = −3.6 + 1.16 x (*Pisano*)	12	0.9912	µmol/l	this article
10*)	y (HPLC) = −3.6 + 1.08 x (*Pisano*)	12	0.9912	µmol/l	this article
11	y (HPLC) = −3.3 + 1.04 x (*Pisano*)	10	0.9259	µmol/24 h	this article
12*)	y (HPLC) = −3.2 + 0.97 x (*Pisano*)	10	0.9267	µmol/24 h	this article
13	y (GLC) = 21.7 + 0.62 x (*Pisano*)	224	0.844	µmol/l	(10)
14	y (GLC) = 2.63 + 1.05 x (*Pisano*)	65	0.997	µmol/24 h	(15)
15	y (HPLC) = 0.61 + 1.06 x (*Pisano*)	41	0.997	µmol/l	(22)
16	y (HPLC) = 0.36 + 0.99 x (*Pisano*)	20	0.931	µmol/24 h	(27)

*) corrected for differences in recovery (see tab. 4).

Tab. 6. Results of the interference study with five methods.

Interfering compound	Concentration (mg/l)	3-Methoxy-4-hydrodroxymandelic acid (µmol/l) GLC	Bio-Rad	*Pisano*	Paper-chromatography	HPLC
None	–	14.9	44.3	25.9	27.7	24.8
3,4-Dihydroxyphenylacetic acid	500	a	13.0	21.3	28.2	23.4
Homovanillic acid	1000	14.8	136.9	9.2[e]	28.4	26.2
p-Hydroxymandelic acid	100	15.2	249.9	52.8	27.4	23.8
Vanillic acid	1000	14.8	46.3	26.7	31.0	24.8
Salicyluric acid	1000	13.2	45.4	27.2	26.7	23.5
Gentisic acid	1000	16.6	47.0	6.2	25.6	26.2
Homogentisic acid	2000	b	43.6	5.6	23.0	27.8[f]
p-Aminosalicylic acid *	1000		40.8		41.8	
5-Hydroxyindoleacetic acid *	500	c	and	21.4	29.8	21.3[g]
Glycerylguaiacolate *	1000		70.3[d]		23.4	
Phenylsulphonephthalein *	6	13.7	43.1	27.5	26.4	23.6
Bromthalein *	350					

*: mixtures of these compounds were added to an aliquot of the pool urine.
a, b: the interfering compound was not separated from 3-methoxy-4-hydroxymandelic acid, because of their large difference in concentration.
c: 5-hydroxyindoleacetic acid was not separated from the internal standard propylgallate, because of the large difference in concentration between 5-hydroxyindoleacetic acid and propylgallate.
d: this difference could not be investigated because the Bio-Rad VMA by Column test was no longer available.
e: 500 mg homovanillic acid gave a result of 7.7 µmol/l 3-methoxy-4-hydroxymandelic acid.
f: 3-methoxy-4-hydroxymandelic acid was not fully separated from an impurity in the homogentisic acid preparation.
g: 5-hydroxyindoleacetic acid (eluting after 3-methoxy-4-hydroxymandelic acid, without interfering) deteriorated the electrode surface, resulting in a decreased detector response for the subsequent analysis.

Discussion

Table 1 shows that differences between the 3-methoxy-4-hydroxymandelic acid concentrations found by the five methods in the same urine sample cannot be attributed to differences in standardization, except for the GLC method. The results of the GLC method indicate that the mean recovery was 90.7%, so all results should be corrected with this percentage. This was only done with the results of the linearity check, the recovery and the comparison study.

The results of the imprecision study (tab. 2) indicate that the within-run and between-days imprecision of the two classical methods are large compared with the other three methods. In the paper chromatographic method, this may be due especially to the sometimes non-optimal separation of 3-methoxy-4-hydroxymandelic acid from very faint coloured spots of unknown compounds. The high coefficient of variation of the *Pisano* method is mainly due to difficulties in determining the urine blanks. In three out of eight series, we found that the absorbance of the urine blanks of the control urine were higher than normal. If a correction is made with the use of a mean urine blank value, we get the imprecision data placed between brackets in table 2. The imprecision data are now comparable with those of the Bio-Rad, the HPLC and the GLC (only within-run CV) methods. The reason for these variations, especially in the urine blank, is unknown.

In the literature we could find data on the imprecision of the *Pisano* method from only one group of investigators: *Wisser & Stamm* (between-days CV = 4.8%; n = 48; $\bar{x}$ = 18 μmol/l; within-run CV = 1.6%; n = 48; $\bar{x}$ = 18 μmol/l) (36). They used a modified *Pisano* method for aliquoting the organic extraction solvents. Therefore we also compared our results with data from a quality control group of clinical chemical laboratories in the Netherlands, *P. J. Brombacher* (37), including laboratories using the original *Pisano* method.

The results of the between-days imprecision of the different methods are given in table 7. Laboratories nos. 6, 7 and 8 performed the *Pisano*, the Bio-Rad and the paper chromatographic method, respectively in the comparison study. Table 6 shows that the mean value of the control urines (aliquoted urine pools, stored at −20 °C) is almost the same for laboratories nos. 1−6, but the ranges of the between-days CV are broad. Both imprecision data, corrected and uncorrected, lie between these ranges, but even the corrected CV is two times higher than that of laboratory 6 in table 7.

Only the corrected results of the imprecision study of the *Pisano* method are comparable with the results of *Wisser & Stamm* (36).

The between-days CV of the GLC method is high. This can be explained by the following facts. The GLC method, not performed in duplicate like the others, is not only a method for the determination of 3-methoxy-4-hydroxymandelic acid, but also of homovanillic acid, 3-methoxy-4-hydroxyphenylethylene glycol, 3,4-dihydroxyphenylacetic acid and 5-hydroxyindoleacetic acid. This means that the analytical conditions are not optimally chosen for the determination of 3-methoxy-4-hydroxymandelic acid; the between-days CV of 3-methoxy-4-hydroxymandelic acid is always higher if the method includes an enzymatic hydrolysis, like the one necessary for the determination of 3-methoxy-4-hydroxyphenyl ethylene glycol, the between-days CV found for 3-methoxy-4-hydroxymandelic acid is in good agreement with that given by *Muskiet* et al. in their original article (14) (CV = 14.3%; n = 20; $\bar{x}$ = 1.6 mmol/mol creatinine).

Tab. 7. Between-days CV of the 3-methoxy-4-hydroxymandelic acid determination in two aliquoted urine pools stored at −20 °C of 8 laboratories participating in a quality control group in the Netherlands, *P. J. Brombacher* (37).

No.	Method	n	$\bar{x}$ (μmol/l)	SD (μmol/l)	CV (%)	n	$\bar{x}$ (μmol/l)	SD (μmol/l)	CV (%)
1	*Pisano*	22	34	6.6	19.5	21	17	3.7	22.6
2	*Pisano*	18	35	2.2	6.4	18	14	1.3	9.3
3	*Pisano*	17	35	2.9	8.2	16	15	1.5	9.6
4	*Pisano*	19	41	4.6	11.0	18	18	2.0	11.0
5	*Pisano*	8	33	4.9	15.0	8	14	1.4	9.8
6	*Pisano*	6	34	1.0	2.9	6	13	0.4	3.2
7	Bio-Rad	31	46	3.4	7.4	31	22	2.4	10.6
8	Paperchromatography	30	33	3.1	9.3	30	15	2.4	15.7

Laboratory nos. 6, 7 and 8 are the same laboratories participating in this comparison study.

The imprecision data of the Bio-Rad and the HPLC method are excellent. The within-run CV of the HPLC method is extremely low (1.4%). Also for the Bio-Rad method a comparison of the imprecision data can only be made with the results of table 7 (laboratory no. 7), showing that the between-days CV is somewhat smaller in our comparison study.

Of those HPLC methods reporting quality control data, only the between-days imprecision of the HPLC method with a periodate oxidative monitor (CV = 5.7 and 6.1%; $\bar{x}$ = 25.0 and 34.5 μmol/l; n = 8 (28)) is comparable with our results. All other HPLC methods (21, 25, 27 and 29) and even the method of *Morrisey & Shihabi* (CV = 7.8%; $\bar{x}$ = 70.0 μmol/l; n = 19 (23)), which was modified, show quality control data with larger imprecision than the HPLC method used in this study.

Moleman & Borstrok (32) publish the following data of their own routine quality control: within-run CV: 3.0; 4.1; 2.0%; between-days CV: 6.5; 4.9; 5.0%; n = 10; 11; 11; $\bar{x}$ = 10.8; 43.2; 74.8 μmol/l. These excellent imprecision data are achieved by the modified sample clean up and by calibrating the detector response and the retention time every six samples.

The results of the linearity check (tab. 3) show a good linearity for all five methods (r ranging from 0.9943—0.9993). However the slopes and the intercepts of the five regression lines are quite different. This means a difference in accuracy between the five methods. In order to investigate whether these differences were due to recovery problems, the same urine sample with low 3-methoxy-4-hydroxymandelic acid content was enriched with known quantities of 3-methoxy-4-hydroxymandelic acid. The results of these recovery studies, presented in table 4, also show a good linearity for all methods (r ranging from 0.9931—0.9994). The mean recovery found with the GLC (95.7%), the *Pisano* (94.4%), the Bio-Rad (96.0%) and the paper chromatographic method (93.9%) are almost equal. The mean recovery found with the HPLC method (101.3%) is somewhat better than the data obtained with the other methods. The HPLC method also shows the smallest standard deviation. These small differences in recovery cannot be responsible for the deviations found in the slopes and intercepts of the regression lines in table 3. Thus, we conclude that the accuracies of the five methods are unequal because of differences in the contribution of urinary compounds other than 3-methoxy-4-hydroxymandelic acid to the determination of 3-methoxy-4-hydroxymandelic acid.

This conclusion is also visualized in the figures 1 a—d presenting the results of the split sample comparison study. Figure 1 a clearly shows that the Bio-Rad method gives higher values, especially in the low concentration range. Sometimes the discrepancy is very large: in one urine sample the HPLC method gives a 3-methoxy-4-hydroxymandelic acid value of 38.5 μmol/l and the Bio-Rad method gives a value of 73.4 μmol/l (mean of four determinations). This means that the contribution to the 3-methoxy-4-hydroxymandelic acid determination of other compounds in urine is very large and may vary from urine to urine. We did not clarify the nature of these compounds.

Figure 1 b and 1 d show that the correlation between the GLC, *Pisano* and HPLC method is reasonable (r = 0.9912 and 0.9929 respectively). Both the GLC and the *Pisano* method give higher values in the high concentration range. Even after correction for the recovery differences (see tab. 4), the *Pisano* method (values above 40 μmol/l) and the GLC method (values above 17 μmol/l) still give lower 3-methoxy-4-hydroxymandelic acid values than the HPLC method (see also the linear regression lines no. 3 and 6 in table 5).

Taking the *Pisano* method as reference method, our split sample comparison results with the GLC method (linear regression line no. 7 in table 5) are better than those of *Addanki* et al. (linear regression line no. 13). Our results are in reasonable agreement with the results of *Leiendecker-Foster & Feier* (see linear regression lines nos. 8 and 14).

The results of *Felice & Kissinger* and of *Bauersfeld* et al., comparing their HPLC method with electrochemical detection with the *Pisano* method as reference method (linear regression lines nos. 15 and 16 respectively in table 5) correlate well with our linear regression lines nos. 10 and 12 respectively.

The correlation between the paper chromatographic method and the HPLC method is the best of all four comparisons (r = 0.9957). However the results of the paper chromatographic method are always higher than those of the HPLC method, but the differences are smaller than with the Bio-Rad method. In our own laboratory we made a more extensive comparison study between the HPLC and the paper chromatographic method using 101 urine samples. This study resulted in almost the same linear regression line: y(paperchrom.) = 3.8 + 1.07 x (HPLC), but with a poorer correlation coefficient: r = 0.9162.

We have taken the HPLC method as reference method for the split sample comparison study because of the good results of the imprecision, linearity, recovery and interference study. However, as can

be seen in figures 1 a–d the 3-methoxy-4-hydroxymandelic acid content of one urine sample indicated with X is overestimated by the HPLC method (14.8 μmol/l) compared with the other four methods (6.2–12.1 μmol/l). This was due to an unknown compound not fully separated from 3-methoxy-4-hydroxymandelic acid.

Compounds which are known to interfere in different 3-methoxy-4-hydroxymandelic acid methods are found in the compilation of *Young* et al. (32). Such a compound can cause an in vivo interference because it intervenes in the catecholamine metabolism or a methodological interference. The physiological interference is of no interest in the comparison of 3-methoxy-4-hydroxymandelic acid methods, so we only studied the methodological interferences. To aliquots of a pool urine, we added those compounds, which, according to the literature, are the real methodological interfering compounds. The amount of each interfering compound added to aliquots of the pool urine was largely based on abnormal levels seen in some disorders.

The results of the interference study (tab. 6) show that the HPLC method is the only method free of interference from the compounds tested. Only 5-hydroxyindoleacetic acid caused some detector problems due to the high concentration, resulting in loss of response. The GLC method sometimes has only separation problems due to too large a difference in concentration between the interfering compound and 3-methoxy-4-hydroxymandelic acid or the internal standard. In cases of pathological increases of 3,4-dihydroxyphenylacetic acid or 5-hydroxyindoleacetic acid, such problems are not encountered, as shown by the gas chromatograms of urines from patients with neuroblastoma and carcinoid tumour in figure 2 of l.c. (14). Only an elevation of homogentisic acid in patients with alcaptonuria could perhaps give some problems in determining 3-methoxy-4-hydroxymandelic acid. The paper chromatographic method is only influenced by *p*-aminosalicylic acid, which produces an elevated background after staining. Correction with a blank including this elevated background prevents falsely elevated 3-methoxy-4-hydroxymandelic acid results.

The *Pisano* and the Bio-Rad method are most sensitive for the interfering compounds tested. The influence of these compounds on the *Pisano* method, mentioned in table 6, are in agreement with the results of *Feldman* et al. (38) and *Pisano* et al. (7). We did not find the decreasing influence of 3,4-dihydroxyphenylacetic acid as reported by *Feldman* et al. Whether the increase of the 3-methoxy-4-hydroxymandelic acid levels obtained by the Bio-Rad method caused by the addition of homovanillic acid and *p*-hydroxymandelic acid, and the decrease of the levels due to the addition of 3,4-dihydroxyphenylacetic acid, are relevant at more physiological concentrations could not be investigated because the Bio-Rad method was no longer available.

If we compare all methods studied, we can conclude that from an analytical point of view the HPLC and the GLC method are superior to the other three methods. Our final conclusion is that the HPLC method is the best method of choice for determining 3-methoxy-4-hydroxymandelic acid in urine, because of the better results of the imprecision study. However this larger imprecision of the 3-methoxy-4-hydroxymandelic acid determination of the GLC catecholamine metabolite profiling method is compensated by the determination of more than one catecholamine metabolite. So it is possible that from a diagnostical point of view this HPLC method, determining only 3-methoxy-4-hydroxymandelic acid, will not be the method of choice. This problem of determining one metabolite very precisely or more metabolites with larger imprecision, which is possible with GLC (14) and HPLC (26) methods, must be the subject of further clinical chemical research.

Acknowledgements

We wish to express our thanks to: *G. T. Berends*, St. Elisabeth's of Groote Gasthuis, Haarlem, The Netherlands; *P. Moleman*, Department of Psychiatry, University Hospital Rotterdam Dijkzigt and Erasmus University, Rotterdam, The Netherlands; *F. A. J. Muskiet*, University Hospital Groningen, The Netherlands and *R. K. A. van Wermeskerken*, Diakonesseninrichting Bronovo, The Hague, The Netherlands, for participating in this comparison study.

The technical assistance of Miss *H. J. Brouwer* in organizing the comparison study is gratefully acknowledged.

References

1. Armstrong, M. D. & McMillan, A. (1957) Fed. Proc. *16*, 146.
2. Armstrong, M. D., McMillan, A. & Shaw, K. N. F. (1957) Biochim. Biophys. Acta *25*, 422–423.
3. Muskiet, F. A. J., Thomasson, C. G., Gerding, A. M., Fremouw-Ottevangers, D. C., Nagel, G. T. & Wolthers, B. G. (1979) Clin. Chem. *25*, 453–460.
4. Armstrong, M. D., Shaw, K. N. F. & Wall, P. E. (1956) J. Biol. Chem. *218*, 293–303.
5. Sandler, M. & Ruthven, C. R. J. (1959) Lancet *II*, 114–115 and 1034.
6. Sunderman, F. W., Jr., Cleveland, P. D., Law, N. C. & Sunderman, F. W. (1960) Amer. J. Clin. Pathol. *34*, 293–312.

7. Pisano, J. J., Crout, J. R. & Abraham, D. (1962), Clin. Chim. Acta 7, 285–291.
8. van de Calseyde, J. F., Scholtis, R. J. H., Schmidt, N. A. & Leyten, C. J. J. A. (1971) Clin. Chim. Acta *32*, 361–366.
9. Roginsky, M. S., Gordon, R. D. & Bennett, M. J. (1974) Clin. Chim. Acta *56*, 261–264.
10. Addanki, S., Hinnenkamp, E. R. & Sotos, J. F. (1976) Clin. Chem. *22*, 310–314.
11. Melchert, H.-U. & Hoffmeister, H. (1977) J. Clin. Chem. Clin. Biochem. *15*, 81–87.
12. Muskiet, F. A. J., Fremouw-Ottevangers, D. C., Wolthers, B. G. & de Vries, J. A. (1977) Clin. Chem. *23*, 863–867.
13. Brewster, M. A., Berry, D. H. & Moriarty, M. (1977) Clin. Chem. *23*, 2247–2249.
14. Muskiet, F. A. J., Stratingh, M. C., Stob, G. J. & Wolthers, B. G. (1981) Clin. Chem. *27*, 223–227.
15. Leiendecker-Foster, C. & Freier, E. F. (1981) Clin. Chem. *27*, 2029–2032.
16. Yoshiue, S., Yoshioka, M. & Tamura, Z. (1975) Chem. Pharm. Bull. *23*, 3040–3041.
17. Chauhan, J. & Darbre, A. (1980) J. Chromatogr. *183*, 391–401.
18. Molnár, I., Horváth, C. & Jatlow, P. (1978) Chromatographia *11*, 260–265.
19. Bertani-Dziedzic, L. M., Krstulovic, A. M., Ciriello, S. & Gitlow, S. E. (1979) J. Chromatogr. *164*, 345–353.
20. Taylor, J. T. & Freeman, S. (1981) Chromatography Newsletter *9*, 1–4.
21. Yamada, K., Kayama, E. & Aizawa, Y. (1981) J. Chromatogr. *223*, 176–178.
22. Felice, R. J. & Kissinger, P. T. (1977) Clin. Chim. Acta *76*, 317–320.
23. Morrisey, J. L. & Shihabi, Z. K. (1979) Clin. Chem. *25*, 2043–2045.
24. Krstulovic, A. M., Zakaria, M., Lohse, K. & Bertani-Dziedzic, L. (1979) J. Chromatogr. *186*, 733–748.
25. Soldin, S. J. & Hill, J. G. (1980) Clin. Chem. *26*, 291–294.
26. Joseph, M. H., Kadam, B. V. & Risby, D. (1981) J. Chromatogr. *226*, 361–368.
27. Bauersfeld, W., Diener, U., Knoll, E., Ratge, D. & Wisser, H. (1982) J. Clin. Chem. Clin. Biochem. *20*, 217–220.
28. Rosano, T. G. & Brown, H. H. (1979) Clin. Chem. *25*, 550–554.
29. Flood, J. G., Granger, M. & McComb, R. B. (1979) Clin. Chem. *25*, 1234–1238.
30. Moleman, P. & Borstrok, J. J. M. (1983) Clin. Chem. *29*, 878–881.
31. Vahidi, A., Roberts, H. R., San Filippo, J., Jr. & Siva Sanhar, D. V. (1971) Clin. Chem. *17*, 903–907.
32. Young, D S., Pestaner, L. C. & Gibberman, V. (1975) Clin. Chem. *21*, 1D-432D.
33. Instruction manual Bio-Rad VMA by Column Test. Catalog 191–8001.
34. Molenaar, A. J. (1963) Boerhavecursus 19, Leiden, Hypertensie, sept. 1963, 310–315.
35. Armitage, P. (1971) Statistical Methods in Medical Research, Blackwell, Oxford.
36. Wisser, H. & Stamm, D. (1970) J. Clin. Chem. Clin. Biochem. *8*, 21–26.
37. Brombacher, P. J. personal communication.
38. Feldman, J. M., Butler, S. S. & Chapman, B. A. (1974) Clin. Chem. *20*, 607–610.

Drs. C. G. Thomasson
Algemeen Ziekenhuis
"de Tjongerschans"
Thialfweg 44
NL-8441 PW Heerenveen